Enhancement of methane production from livestock manure with pre-treatments based in fungi of genus *Pleurotus*

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ABSTRACT

Livestock manure, traditionally used just as fertilizer, can be energetically valued to produce biogas as an attractive alternative, since nowadays, energy production and its cost stands for a pressing problem around the world. Nevertheless, the presence of lignin in manure hinders the production of methane. This could be improved by pre-treating the manure with ligninolytic fungi, able to break lignin and therefore facilitate the hydrolysis step for the hydrolytic bacteria, yielding higher volumes of biomethane. Three strategies of incubation with living fungi of genus *Pleurotus* were evaluated to enhance methane production from livestock manure mixed with bedding material: short term (two weeks 2L container) and long term (two months 400 L container) and 24 h (2 L container) with a crude water extraction of *Pleurotus* extracellular enzymes. The positive effect of the fungal treatment was observed in the three strategies obtaining an increase in methane production with respect to the control manure of 7% at short term, 111% at long term and 173% (crude enzymatic extract). Consequently, the strategy of using crude enzyme extracts from *Pleurotus* to improve hydrolysis step as pre-treatment of manure should be considered as a novel, easy, cheap and promising tool to optimize methane production.

1. Introduction

The management of livestock wastes is considered a key problem because of the enormous quantities produced all over the world, but at the same time it is an inexhaustible source of nutrients and energy which should not be managed as waste, especially in view of Europe's energy-dependent situation, particularly aggravated by the climate change. Therefore, the search for alternative resources is of utmost importance to overcome dependency on fossil energy [1].

The most common management of manure is its use as organic fertilizer into agricultural soils. However, its uncontrolled use is responsible of soil pollution [2], groundwater contamination by infiltration [3], or uncontrolled greenhouse gas emissions (mainly CH_4 and NH_3) that should be avoided [4]. For a long time, anaerobic digestion (AD) has been an optimal strategy to handle organic wastes [5], becoming an alternative to waste disposal as well as a renewable energy source [6]. However, biogas production is highly variable, since the amount and composition of methane produced depend on the type of waste, the inoculum used and the design of the facilities [7]. Cattle manure, which is widely produced, is usually partially converted in methane due to the high content of lignocellulosic material (40–60%) [8]. Several researchers enhance methane yields when mixing cattle manure with some lignocellulosic materials, such as the case of wheat straw, commonly used for animal bedding, which show high potential for producing methane but should be pre-treated to turn hemicellulose and cellulose into soluble compounds [9], sugar beet by-products [10], or cocoa wastes [11]. The presence of lignin hinders the hydrolysis stage of the process [12]. Thus, lignocellulosic materials, although very abundant, are not widely used in AD despite its high content in carbohydrates. Its recalcitrant nature handles poor methane yields, due to the low hydrolysis rate caused by the presence of lignin, which combined with cellulose and hemicellulose units forms a rigid three-dimensional complex compound that is exceedingly difficult to breakdown by bacterial enzymatic attack [13]. Hydrolytic bacteria, mostly belong to phyla

Firmicutes and *Bacteroidetes,* not always are able to break down the plant cell wall. However, some bacteria from genus *Clostridium* are known for being able to degrade lignocellulosic biomass by secreting multienzyme complexes named cellulosomes which contribute to degrading the plant cell wall, mainly cellulose and hemicellulose [14], but not lignin. Cellulose crystallinity also contributes to the hardness of the cell wall and slows down its degradation. So, lignin needs to be broken down and cellulose and hemicellulose (holocellulose) decomposed to increase the methane yield. Therefore, a pre-treatment of the lignocellulosic materials with ligninolytic fungi could enhance the rate of hydrolysis by means of extracellular enzymes like laccase (Lac), Manganese peroxidase (MnP) or Lignin peroxidase (LiP). Some lignin-modified fungi (LMF) degrade selectively lignin and hemicellulose fraction and slightly affecting cellulose like *Pleurotus ostreatus* or *Pleurotus eryngii*, meanwhile others are nonselective fungi, breaking down all the fractions including cellulose [15], such as the case of *Trametes versicolor,* which usually consumes carbohydrates faster than the selective ones, so a loss of nutrients for bacteria occurs [16]. In general, microbial pre-treatments are very attractive, cost-effective and environmentally friendly methods to increase the digestibility of the substrate however, they are time consuming [17].

Some authors have reported increases in methane yield after a pre-treatment of lignocellulosic materials with different species of ligninolytic fungi, such as *P. ostreatus, Phanerochaete chrysosposrium*, and *Ganoderma lucidum* [18]; *P. ostreatus and Trichoderma reesei* yielded 96 and 60%, respectively, more methane than raw rice straw with an optimal moisture content of 75% [19]; and *Pleurotus eryngii*, which improved methane yield 19% while *P. ostreatus* and *T. versicolor* not [16]. The use of enzymes to break down lignin reduced the period of pre-treatment, which could be a valuable approach as some authors suggest. [20] tested three commercial enzymes (cellulase, protease and amylase) and mixtures of them, to improve the production of methane from the microalgae *Chlorella vulgaris* as feeding for AD, resulting in increasing of methane yields by 22–162%. Another example is [12], who used commercial fungal ligninolytic enzymes, like laccase from *T. versicolor* and versatile peroxidase from *Bjerkandera adusta* as pre-treatment on several lignocellulosic material before AD. They performed the enzymatic pre-treatment for 6h and 24h, resulting in no difference on the measured parameters, remarking the importance of breaking lignin to improve methane yield although using commercial enzymes is an expensive pre-treatment. However, lignin content of the substrate is not the only parameter that determines the production of methane. Temperature is known to be a key factor for AD process, being

a general rule that the performance of the process is increased with the increase in temperature [21]. Another key parameter is the kind of inoculum since the anaerobic bacterial community is characteristic of each one [22].

Methane production studied by Biochemical Methane Potential (BMP) assays, have been thoroughly found to produce reasonable predictions of full-scale behaviour [23]. Some authors compared the volume of methane predicted by BMP data with the methane production measured onsite from a full-scale installation for 7–9 months, finding that the weekly methane production rates from BMP were similar to the full-scale rates and followed the same pattern [24].

The aim of this work was to assess the effect on methane yield of the pre-treatment for two weeks (short-term assay) and two months (long-term assay) of dairy cattle manure with living fungi of genus *Pleurotus*. Both pre-treatments were compared with a 24 h incubation of the manure with a crude water extract obtained from *Pleurotus* spawn. For this purpose, anaerobic digestion was carried out by Biochemical Methane Potential (BMP) assays [14].

2. Materials and methods

2.1. Inoculum and substrate

Dairy cattle manure was collected mixed with the bedding material, composed mainly of wheat straw, from a livestock farm located in Burgos province 41° 40′ 18″ N, 3° 41′ 12″ W (Spain). The manure and the bedding material were mechanically homogenised using a hand blender (Braun 7 MQ 7045X) for the lab scale assay. Sludge from an anaerobic digester located in the same farm was used as the inoculum of the biomethanization process.

Wheat-based spawn of *P. ostreatus* was purchased from Gurelan Mycelium (Huarte, Navarra, Spain).

2.2. Characterization of the substrate

The organic matter (OM) content was determined by calcination (550 °C, 1 h) after its dehydration (105 °C, 24 h). The contents in C, H, N, and S of the organic materials were determined by elemental analysis (LECO CHNS-932, St. Joseph, MI, USA). The oxygen content was calculated as the difference between 100 and the sum of C, N, S and H percentages.

2.3. Theoretical biochemical methane potential (TBMP)

The theoretical biochemical methane potential (TBMP) of the cattle manure sample was estimated from its elemental analysis according to the formulas reported by [27] modified by Boyle, and [28], shown in equations (1) and (2). It means a theoretical approach to the potential of a substrate to produce CH_4 under ideal conditions, considering that the reaction goes to completion. A factor f (=80%) was applied to the value obtained from equation (2) to make results more reliable and realistic because in real conditions, the whole substrate is not converted to biomethane, according to [29].

$$C_aH_bO_cN_dS_e + \left(a - \frac{b}{4} - \frac{c}{2} + \frac{3d}{4} + \frac{e}{2}\right)H_2 \rightarrow \left(\frac{a}{2} + \frac{b}{8} - \frac{c}{4} - \frac{3d}{8} - \frac{e}{4}\right)CH_4 + \left(\frac{a}{2} - \frac{b}{8} + \frac{c}{4} + \frac{3d}{8} + \frac{e}{4}\right)CO_2 + dNH_3 + eH_2S \quad (1)$$

$$TBMP\left(mLCH_4/g\ VS\right) = \frac{22.4 \times \left(\frac{a}{2} + \frac{b}{8} - \frac{c}{4} - \frac{3d}{8} - \frac{e}{4}\right)}{12a + b + 16c + 14d + 32e} \quad (2)$$

The coefficients a (C), b (H), c (O), d (N) and e (S) represent the elemental analysis-based mass divided by the molar mass of the element.

The degradability percentage of the substrate was determined as the ratio between the experimental and the theoretical methane yield.

Table 1
Percentage of Total solids (TS), volatile solids (VS, and VS/TS) and soluble chemical oxygen demand (SCOD) in gO2/L of the substrates and inoculum. Results are the mean ± standard deviation (n = 3). Different letters show significant differences among treatments (Duncan post-hoc test; $p \leq 0.05$).

	TS	VS	VS/TS	SCOD
Short-term assay				
Inoculum	3.1 ± 0.1[a]	2.0 ± 0.1[a]	66±2[a]	1.2 ± 0.1[a]
Manure	12.1 ± 0.5[b]	10.0 ± 0.2[b]	83±1[b]	8±1[b]
Manure + *P. ostreatus*	13.9 ± 0.3[c]	11.4 ± 0.3[c]	81.9 ± 0.4[b]	8±1[b]
Enzymatic extract	16.5 ± 0.2[e]	14.8 ± 0.3[d]	89±1[c]	3.8 ± 0.2
Long-term assay				
Inoculum	3.3 ± 0.1[a]	2.2 ± 0.1[a]	67±1[a]	1.9±4[a]
Manure	11.8 ± 0.2[b]	9.9 ± 0.1[b]	84±1[b]	8±1[b]
Upper layer + *P. ostreatus*	42±1[c]	35±1[b]	82.7 ± 0.3[b]	32±1[c]
Lower Layer + *P. ostreatus*	12.2 ± 0.1[b]	9.9 ± 0.1[c]	84±1[c]	34.2 ± 0.1[d]

2.4. Pre-treatment of the manure before anaerobic digestion

Two different kinds of fungal pre-treatments were performed in this work prior to the anaerobic digestion of the manure: the inoculation of the living fungus and the enzymatic crude extract.

The pre-treatment based in inoculation of the living fungus was performed according to two different set-up based on time.

- On one hand, in the short-term assay, the homogenised manure was dried at room temperature for 24h. Then, it was inoculated with spawn of *P. ostreatus* in a ratio manure/spawn 80/20 w/w in containers of 2.5 L and incubated at 28 °C in darkness for two weeks.
- In the long-term assay, a 400 L container simulating a pile of manure in a farm, with non-favoured leaching conditions was filled with manure 2 layers of 25 cm height and in between and in the top inoculated with spawn of *P. ostreatus*. The container was covered with a black canvas to avoid light and moisture loss and placed inside a greenhouse at temperature ranged between 2 and 17 °C minimum to 10–31 °C maximum between March and May 2020. The pile was settled for two months. The pile showed a clear stratification of the manure into two different levels that were carefully separated: the upper layer (0–25 cm), which was darker brown and seemed drier, and the lower layer (25–50 cm) whose colour was yellowish and showed some liquid at the bottom. An aliquot (1 kg) of each layer was taken and underwent an anaerobic digestion process in a BMP assay. 1 kg of manure was stored at 4 °C when the pile was set to be used as a control.

The pre-treatment based on the incubation of the manure with the enzymatic crude extract was performed after a previous selection of *Pleurotus ostreatus and Pleurotus eryngii* species. The extracts were prepared with distilled water (1:1) and after 1h of orbital shaking, then the aqueous extracts were measured for laccase activity, selecting the one with higher enzymatic activity: *P. eryngii* (3 U/mL). Then, the extract was poured homogenously over the homogenised manure (100 mL on 400 g of manure) and kept for 24 h at 28 °C in the dark. Afterwards the manure was introduced in the digesters.

2.5. Anaerobic digestion (AD)

Anaerobic digestions took place in micro digesters (500 mL) in batch on discontinuous basis in triplicate at 37 °C. The product of AD is biogas, which typically consists of methane (50–75%), carbon dioxide (25–50%), and smaller amounts of nitrogen (2–8%) and traces of SH_2, ammonia and hydrogen [30].The methane generated was volumetrically measured by using a 15-channel volume displacement measuring cell unit after passing the biogas stream through a NaOH trap to remove all the CO_2 by a unit AMPTS II (Bioprocess Control, Lund, Sweden). The ratio inoculum: substrate was 2:1 (w:w) based on their volatile solids (VS).

Two sets of AD assays were performed, one for the manure pre-treated with the living fungus at short-term with the crude enzymatic extract treatment and another one for the long-term assay (400 L container). In both assays, a double control was performed, one with the inoculum and the other one with untreated manure. The BMP assays were considered finished when the methane production was less than 1% of the previous day.

The substrates for the first AD assay were: Inoculum (control 1), untreated manure (control 2), manure pre-treated with *P. ostreatus* for 14 days and manure pre-treated with the enzymatic extract for 24 h.

The substrates for the second AD assay were: Inoculum (control 1), untreated manure (control 2), manure pre-treated with *P. ostreatus* for 2 months upper layer (0–25 cm) and lower layer (25–50 cm).

The control parameters were VS performed using a furnace Digitronic NCC-160 (J P. Selecta, Barcelona, Spain) and a muffle Select-Horn, (J.P. Selecta, Barcelona, Spain) [31], total (COD) and soluble Chemical Oxygen Demand (SCOD) quantified by colorimetry using a spectrophotometer of Hanna instruments (Smithfield, RI 02917 USA) [32]. Total Kjeldhal nitrogen (TKN) and ammonia nitrogen by titration using KjelFlex K-360 coupled with TitrinoPlus (Büchi Labortechnik, Flawil, Switzerland), total (TA), partial (PA), and intermediate alkalinity (IA) [33] and volatile fatty acids (VFA) [34].

2.6. Statistical analysis

Statistical analysis was carried out by one-way analysis of variance (ANOVA). Duncan post hoc test at $p < 0.05$ was used to determine significant differences between treatments. All statistical tests were carried out using the IBM SPSS Statistics v25 software package. The principal component analysis (PCA) was performed to determine the relation between the production of methane and the control parameters of the process. This test was done using the PAST V. 4.02 software (Natural History Museum, University of Oslo).

3. Results and discussion

3.1. Physico-chemical characterization of the substrate and the inoculum

After the physico-chemical characterization of the manure (n = 3) the mean percentages ± standard deviations of C, N, H and S were 41.7 ± 0.2; 1.28 ± 0.02; 5.59 ± 0.03 and 0.47 ± 0.07 respectively with a C/N ratio of 33 ± 1, close to the suitable one for AD, since many studies stated that the optimal C/N ratio for AD should be between 20 and 30. The organic matter (OM), total solids (TS) and volatile solids (VS) were 82 ± 3; 12.1 ± 0.5 and 10.0 ± 0.2 respectively.

Total solid (TS) is a parameter used to determine the water content of the substrate, represent the dry matter content and could be an inhibitor factor since the mixing operations inside the digesters is highly dependent on the dry matter content, being the optimal values in the range of 2%–10% [35]. VS is a parameter used to determine the organic loading rate of the digesters.

Table 1 shows TS, VS, VS/TS and soluble chemical oxygen demand (SCOD) of the inoculum and the substrates used in the two AD assays before being introduced in the digesters. In short-term, the enzymatic extract pre-treatment of the manure showed significantly higher VS than the control, which could mean a higher degradability of the substrate due to the rate of delignification performed by the fungal enzymes, since the ability of ligninolytic fungi and their extracellular enzymes to break down lignin has been thoroughly proven for a long time [15]. However, the contribution of fungal spawn to methane production when inoculated on manure in both assays was negligible as the manure/fungal spawn ratio was low (80/20 w/w) and after the incubation period, the fungus would have consumed most of the nutrients of the cereal grain to grow and develop. In long-term assay, the manure of the upper layer (0–25 cm) showed a significant increase in TS and VS compared to the control, due to the decrease of water produced by higher evaporation. The higher dryness reduced the mineralization of the organic matter. In contrast, the lower layer (25–50 cm deep) showed no significant differences compared to the control in TS and VS It is remarkable the significant higher values of SCOD observed in both layers: upper (32 ± 1 gO_2/L) and lower (34.2 ± 0.1 gO_2/L) in relation to the control, which could be explained by the fungal performance together with the storage conditions during the two months before the AD.

3.2. Methane production after the pre-treatment with ligninolytic fungi

3.2.1. Short-term pre-treatment assay

All the initial and final control operational parameters of the mixture inoculum/substrate (ratio 2:1 w/w VS) in the digesters were stable along the AD, as shown in Table 2. The initial pH was higher than the optimum for methanogens (6.6–7.5), keeping in the same range until the end of the digestion. However, at the end of the digestion, the pH was slightly

lower in every digester compared to the initial value. This fact might be due to the observed increase in VFAs at the end of AD possibly as a consequence of the acidogenic bacteria, usually quicker than methanogenic archaea, which did not have enough time to transform those VFAs in methane. Methanogens show a poor adaptation to the variation of pH [39], but [40] reported an increment of the activity of some families of acetotrophic methanogens like *Methanosarcinaceae* and *Methanosaetaceae*, resulting in the enhancement of methane production via consumption of acetic and formic acid and hydrogen after an increase of pH. [39,40] also stated that a hydrogenotrophic genus, *Methanobacterium*, which utilizes carbon dioxide and hydrogen to generate methane, can be inhibited in an infrequently low pH. This reduction of pH is normally compensated by the methanogenic bacteria through the production of CO_2, ammonia and bicarbonate [41]. Regarding VFAs, the manure pre-treated with the enzymatic extract, initially showed 1.8 folds more VFAs than control. On the other hand, the manure pre-treated with the living fungus did not have a remarkable increase in VFAs. Initial VFAs might be a parameter to assess the performance of the fungal enzymes as [18] suggested, since the degradation products produced in pre-treatment are transformed into VFAs, which later would be transformed in acetate and then converted in methane by the acetoclastic methanogens. But if VFAs were accumulated in the digesters could inhibited the methane production, so [42] suggested that the ratio intermediate alkalinity/partial alkalinity (IA/PA) in a digester operating with poultry manure must be below 0.3, which could give an idea of the buffering capacity and the accumulation of VFAs in the digesters. In this assay, all the digesters showed a ratio IA/PA $\leq$ 0.3 as can be seen in Table 2, which means that the digesters were successfully working.

The methane production can be seen in Fig. 1A and B, which shows the cumulated and daily production per gram of load VS. The curves show the volumes of methane produced by manure and manure treated with *P. ostreatus* or the enzymatic extract. It is remarkable the cumulated methane production of the enzymatic pre-treatment (295 mL CH_4 VS^{-1}), which was 173% higher than the control meanwhile the fungal treatment significantly improved ($p < 0.05$) the methane production of control 5%, lower than expected. Comparing the experimental results with the theoretical biomethane production (TBMP), which is a simplistic model to figure out the theoretical total amount of biomethane that could be produced from cattle manure by AD, it turned out that the enzymatic pre-treatment produced a cumulative volume of methane very close to the theoretical one, based on the elemental analysis (300 mL CH_4/g VS), reaching a degradability of 98%, while the living fungal pre-treatment and the control did not show a degradability higher than 40% of the theoretical one (Table 3). AD under favourable conditions of manure might reach a conversion into methane between 30 and 60%, which is considered normal by some researchers [43], meanwhile substrates with mainly soluble organic matter could achieve up to 90–95% of conversion. During AD, approximately 10% of the

substrate is used for bacterial growth and transformation into heat [44]. Therefore, the pre-treatment with the enzymatic extract successfully improved the biodegradability of the manure with the bedding material, reaching a methane yield very close to the theoretical one. Beforehand, the TBMP from the substrate could give an idea of the biomethane that might be obtained from a certain material assuming the total conversion of the substrate in methane. As expected, most of the samples yielded lower methane than the theoretical one, which are consistent with the results obtained by [29], also in samples of different manures.

The reason for those increments in the production of CH_4 might be the capability of the fungal extracellular enzymes to delignify the lignocellulosic materials, which allowed better access of hydrolytic bacteria to nutrients, resulting significantly more efficient when the manure was treated with the aqueous fungal enzymatic extract than the fungus itself. In addition to be cheap and environmentally friendly, it reduced the pre-treatment period to 24 h, which is a great advantage, thus more research should be done in this respect considering that fungal incubation period represents a bottleneck to scale up to industrial level, in addition to its dependence on the fungal species and the nature of lignocellulosic biomass [45]. Earlier works pre-treated different lignocellulosic wastes with commercial laccase from *T. versicolor* (2 U/g substrate) mixed with versatile peroxidase (VP) from *Bjerkandera adusta* (1.5 U/g substrate) resulting that the period of the enzymatic pre-treatment had no significant impact, stating the effect of enzymatic pre-treatment occurred in the first hours of incubation and stabilized afterwards. Methane production was increased compared to the control in some of the materials like corn stover or flax, concluding that the kind of substrate is a crucial factor since each one behaved different. In addition, lignin should be broken before anaerobic digestion of any lignocellulosic substrate and ligninolytic enzymes might be a good option, but the leaking of phenolic compounds should be studied as possible inhibitors of AD [12]. Considering the results of *P. ostreatus* in this assay, a period of 14 days seemed to be suitable although not optimal since the increase in methane production was not as high as expected. Fungal pre-treatment time is an important parameter to get a positive or negative effect on the methane yield as fungi also feed from the substrate. However, this parameter seems to be controversial as shown the results obtained by different researchers. [19] obtained better results after 20 days of incubation of *P. ostreatus* and *T. reesei* on rice straw than after 30 days, with increases of more than 70% over the control in cumulative methane, and a selective lignin removal of 33.4% was found with *P. ostreatus*. They also stated that apart from the fungal incubation time, moisture content also significantly affected the efficiency of fungal pre-treatment of rice straw by both fungi with respect to lignin removal. Meanwhile [16] found a negative effect on methane generation potential of corn stover when it was pre-treated with *P. ostreatus* for 30 days while *P. eryngii* showed an increase of 19% compared with raw waste after the same incubation period. [46]

Table 2
Initial and final parameters of the mixture of inoculum and substrate digesters in the first anaerobic digestion assay: Total solids (TS), volatile solids (VS), VS/TS, pH, volatile fatty acids (VFA), total alkalinity (TA), ratio intermediate alkalinity (IA)/partial alkalinity (PA) (IA/PA), ammonia nitrogen, Total Kjeldhal nitrogen (TKN), and total (COD) and soluble chemical oxygen demand (SCOD). Different letters show significant differences among treatments (Duncan post-hoc test; $p \leq 0.05$).

| Parameter	Initial parameters				Final parameters			
	Inoculum	Control	*P. ostreatus*	Enzymatic extract	Inoculum	Control	*P. ostreatus*	Enzymatic extract
TS (%)	2.99 ± 0.01[a]	3.6 ± 0.6[ab]	4.17 ± 0.03[b]	3.0 ± 0.1[a]	3.9 ± 0.1[bc]	3.3 ± 0.5[bc]	3.9 ± 0.2[c]	2.8 ± 0.1[a]
VS (%)	1.77 ± 0.04[a]	2.6 ± 0.3[ab]	2.75 ± 0.02[b]	2.3 ± 0.2[ab]	2.32 ± 0.02[a]	2.3 ± 0.2[ab]	2.6 ± 0.2[b]	2.1 ± 0.2[a]
VS/TS	50 ± 1	71 ± 4	66 ± 1	80 ± 4	60±1[a]	68±4[b]	66±1[b]	75±4[c]
pH	8.0 ± 0.1[ab]	7.9 ± 0.4[bc]	8.1 ± 0.1[abc]	8.2 ± 0.2[d]	7.7 ± 0.1[a]	7.7 ± 0.2[a]	7.64 ± 0.03[a]	8.1 ± 0.2[b]
VFA mg AcH/L	0.21 ± 0.00[a]	0.27 ± 0.04[ab]	0.29 ± 0.05[ab]	0.5 ± 0.0[c]	0.3 ± 0.1[ab]	0.5 ± 0.1[b]	0.33 ± 0.02[ab]	0.47 ± 0.05[b]
TA (mg CaO₃/L)	517 ± 14	520 ± 59	567 ± 52	528 ± 64	558 ± 14[a]	609 ± 33[b]	633 ± 14[b]	700 ± 33[c]
IA/PA	0.19 ± 0.05[a]	0.2 ± 0.1[a]	0.3 ± 0.1[c]	0.3 ± 0.1[bc]	0.155 ± 0.005[ab]	0.3 ± 0.1[c]	0.27 ± 0.03[bc]	0.11 ± 0.01[a]
NH₃–N %	0.26 ± 0.00[c]	0.25 ± 0.05[b]	0.22 ± 0.02[b]	0.15 ± 0.01[a]	0.26 ± 0.01[b]	0.21 ± 0.03[b]	0.25 ± 0.00[b]	0.19 ± 0.00[a]
TKN %	0.41 ± 0.02[c]	0.34 ± 0.04[b]	0.41 ± 0.01[c]	0.19 ± 0.00[a]	0.36 ± 0.01[b]	0.37 ± 0.04[c]	0.41 ± 0.01[d]	0.35 ± 0.01[a]
COD g/L	23±1[a]	32±5[ab]	31±1[b]	33±2[b]	19±2[a]	21±6[b]	33±4[c]	30±3[bc]
SCOD g/L	1.1 ± 0.1[a]	3±1[a]	1.9 ± 0.1[a]	3.0 ± 0.2[b]	0.878 ± 0.003[a]	1.1 ± 0.3[a]	2.1 ± 0.1[b]	4.1 ± 0.2[c]
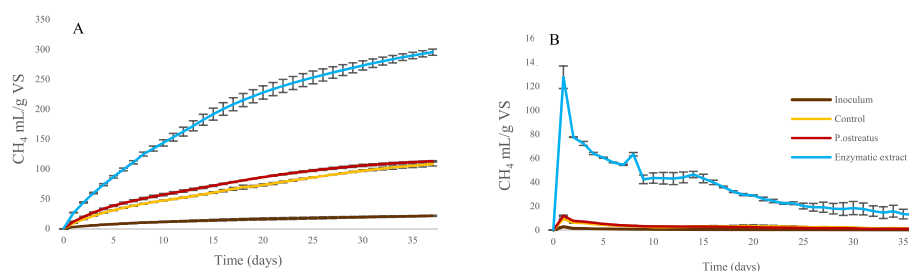
Fig. 1. Methane production in short-term assay: A: Cumulated production, B: daily methane production (n = 3).

Table 3
Experimental methane yield (mL CH4 g VS-1), and degradability of the substrates of AD (%) vs TBMP of the manure in short- and long-term assays.

	Samples	Experimental CH$_4$ yield	Degradability %
Short-term assay	Control	106 ± 3	35
	Fungal pre-treatment	113 ± 1	38
	Enzymatic extract	295 ± 5	98
Long-term assay	Control	118 ± 6	39
	Upper layer	114 ± 4	39
	Lower layer	249 ± 12	83

reported a faster degradation of cellulose than lignin by *P. eryngii* during the pre-treatment of wheat straw which resulted in a negative effect on methane production, meanwhile *P. ostreatus* showed 25% of increment over the control after 15 days of treatment. [47] reported that *P. ostreatus* after 60 days of incubation on rice straw lowered methane production since cellulose and hemicellulose contents resulted also reduced. [48] pre-treated chicken manure mixed with sawdust and barley straw at different proportions with *P. ostreatus* or *T. versicolor*, concluding that the fungal pre-treatment did not show the expected results in methane yield.

The positive effect of the fungal pre-treatment on hydrolysis was observed from the first day with the enzymatic pre-treatment meaning a much higher bioavailability of the nutrients probably due to the breakdown of lignin, producing 67% more methane than control that first day. Enzymatic pre-treatment seemed to facilitate the first stage of the process by making it faster since the rest of the samples, included the one of living fungus pre-treatment, reached their maximum of methane production at day 2. Fungal performance achieved 25% more methane than the control (Fig. 1B). The digesters corresponding to enzymatic pre-treatment continued producing, along the digestion period, higher volumes of methane than those corresponding to control and fungal pre-treatment. The fungal pre-treatment digesters also continued yielding more methane than control until day 14th, since nutrients were more readily available after fungal pre-treatment and bacteria consumed them faster than in manure (control). The increase of easy degradable compounds and soluble organic matter (SCOD) produced higher biomethane yield from the same substrate in the treatment with the enzymatic extract. These results were obtained using another strategy to enhance methane production like the co-digestion of manure and other materials with lignocellulosic substrates, improving some parameters such as C/N or the content in soluble organic matter, stating the importance of soluble organic matter along the AD to methane production [49]. [19] reported high levels of delignification of rice straw by *P. ostreatus* and low rate of degradation of cellulose which is considered the main substrate for anaerobic microorganisms to produce methane by AD. The fungus consumed digestible sugar fractions, and as the time of pre-treatment became longer total soluble sugar and glucose increased progressively. It seems difficult to evaluate the effect of incubation time on the substrate methane production, since it might be related with fungus characteristics and the nature of substrate [50], so those

researchers reported selective white rot fungi (WRF) like *P. ostreatus* and *Dichomitus squalens*, which attack lignin and hemicellulose and do not much affect cellulose, showed a positive effect on methane production, meanwhile non-selective fungi like *T. versicolor* or *Irpex lacteus* had a negative effect on AD efficiency.

A PCA analysis was done to study the correlation of the control parameters and the methane production, as can be seen in Fig. 2. The digesters with the enzymatic pre-treatment were strongly related with methane production and some key parameters like COD, SCOD, VFAs and pH, while the rest of the samples were not. However, the relation of enzymatic pre-treatment with the COD and SCOD parameters was more noticeable at the initial time (T0) than at the final (TF) which correlated to the increment in the degradability of the lignocellulosic fraction of the substrate by the enzymatic extract. *P. ostreatus* T0 was related with TS and VS at the initial time but at the end (TF) this group was related more with TKN and NH$_4^+$ indicating that methane production may have been inhibited to some extent by the concentration NH$_3$/NH$_4^+$ in the digesters at the end of the assay [51].

Inoculum and control groups did not vary the relation with the parameters (mainly related to TA and negatively related with COD and SCOD) over the length of the assay as it can be seen in Fig. 2.

3.2.2. Long-term pre-treatment assay

The scale in this assay and the period of pre-treatment in a container of 400 L tried to emulate the conditions of the storage of the manure in the farms. In addition, this assay had the objective of assessing the effect of that storage conditions together with the fungal pre-treatment of the manure mixed with the bedding material could have on methane production. The assay operational parameters are shown in Table 4.The fungal pre-treatment, the storage conditions and all the processes that could have occurred led to some differences in some parameters among the two layers of the mycopile. SCOD is an important parameter greatly increased in the lower layer with respect to the control and the upper layer at the initial point. The rupture of the lignocellulosic cell wall of the straw might have released organic micro-molecules in the soluble phase [18]. It was two folds higher than the control and 1,7-folds than the upper layer. This fact could explain the higher methane yield together with the VFAs, whose initial concentration in the lower layer was 2.5-folds and 2-folds higher than the control and upper layer respectively. It might be the result of all the processes that occurred during the two months of storage together with the fungal pre-treatment. Therefore, like occurred in short-term assay with the enzymatic pre-treatment, the production of methane was higher (Fig. 3). However, the decrease in SCOD throughout the AD was similar to the upper level showing a 76 and 78% respectively, being lower in control (70%). Both parameters SCOD and VFA have a key role in the overall methane production [39]. pH were close to optimum values for AD along the assay in all the digesters [39] and the behaviour of the total alkalinity was similar to the short-term assay (Table 3 and Table 4), however the lower level digesters showed a IA/PA ratio higher than 0.3 at the initial time that was corrected at the final stage. The efficiency in VS removal was also the highest in the lower layer, 48% higher than the control and 60% than the upper layer, which meant anaerobic bacteria
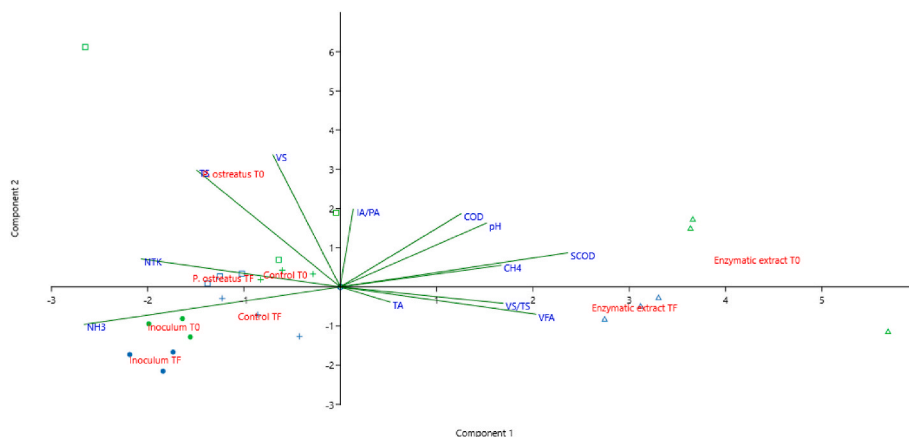
Fig. 2. PCA analysis of the studied parameters in short-term assay at initial (T0, marked in green) and final time (TF marked in blue) depending on their group: Inoculum (●), Control (+), manure pre-treated with P.ostreatus (□), and manure pre-treated with Enzyme (△). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 4

Initial and final parameters of the digesters of anaerobic digestion (AD) in long-term assay: Total solids (TS), volatile solids (VS), VS/TS, volatile fatty acids (VFA), total alkalinity (TA), ratio intermediate alkalinity (IA)/partial alkalinity (PA) (IA/PA), Total Kjeldhal nitrogen (TKN), ammonia nitrogen and total (COD) and soluble chemical oxygen demand (SCOD). Different letters show significant differences among treatments (Duncan post-hoc test; $p \leq 0.05$).

|	Initial parameters				Final parameters			
Parameter	Inoculum	Control	Upper level	Lower level	Inoculum	Control	Upper level	Lower level
TS (%)	3.34 ± 0.02^a	3.6 ± 0.6^b	4.6 ± 0.2^c	3.96 ± 0.02^b	3.12 ± 0.02	3.3 ± 0.5	4.07 ± 0.04	3.38 ± 0.01
VS (%)	2.24 ± 0.02^a	2.6 ± 0.3^c	3.37 ± 0.03^d	2.81 ± 0.05^b	1.8 ± 0.1^a	2.3 ± 0.2^{bc}	2.78 ± 0.04^c	2.20 ± 0.03^b
VS/TS	67.0 ± 0.3^a	71 ± 4^b	73 ± 3^b	71 ± 1^b	59 ± 4	68 ± 4	68.4 ± 0.3	65 ± 1
pH	7.8 ± 0.1^b	7.9 ± 0.4^a	7.55 ± 0.03^a	7.4 ± 0.1^a	8.0 ± 0.2^b	7.7 ± 0.2^{ab}	7.7 ± 0.1^{ab}	7.7 ± 0.1^a
VFA (mg AcH/L)	0.23 ± 0.02^a	0.27 ± 0.04^a	0.36 ± 0.04^b	0.707 ± 0.003^c	0.23 ± 0.03	0.5 ± 0.1	0.4 ± 0.1	0.37 ± 0.02
TA (mg CaO$_3$/L)	450 ± 25^a	467 ± 14^{ab}	492 ± 14^b	483 ± 14^{ab}	533 ± 38^a	583 ± 14^{ab}	608 ± 38^b	617 ± 14^b
IA/PA	0.27 ± 0.05^a	0.3 ± 0.1^{ab}	0.31 ± 0.04^{ab}	0.42 ± 0.05^b	0.21 ± 0.03	0.18 ± 0.04	0.22 ± 0.02	0.30 ± 0.04
NH$_3$–N %	0.30 ± 0.01	520 ± 59	0.28 ± 0.02	0.3 ± 0.1	0.20 ± 0.01^c	0.20 ± 0.01^c	0.19 ± 0.01^b	0.18 ± 0.00^a
TKN %	0.43 ± 0.0	0.25 ± 0.05	0.47 ± 0.00	0.44 ± 0.01	0.44 ± 0.02^c	0.41 ± 0.01^b	0.37 ± 0.00^b	0.39 ± 0.01^a
COD g/L	26.9 ± 0.5^a	37 ± 1^b	36 ± 1^b	35 ± 2^b	11.2 ± 0.1^a	15 ± 1^b	15 ± 1^b	12 ± 1^a
SCOD g/L	1.9 ± 0.1^a	2.6 ± 0.1^b	2.8 ± 0.1^b	5.0 ± 0.2^c	0.6 ± 0.1^a	0.77 ± 0.03^a	0.6 ± 0.2^a	1.2 ± 0.2^b
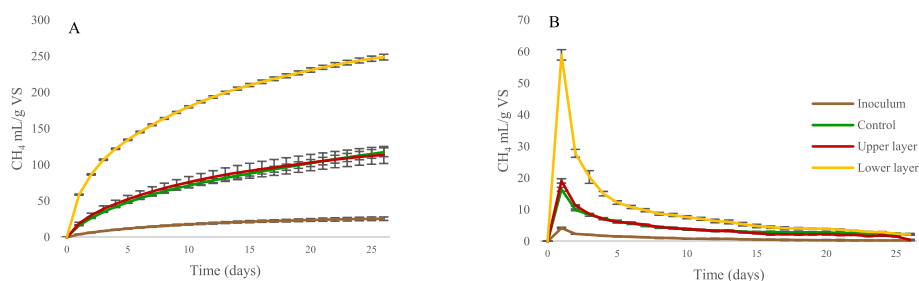
Fig. 3. Methane production in the long-term assay: A: cumulated production mL CH$_4$ gVS-1, B: Daily production (mL CH$_4$ gVS-1). (n = 3).

were more efficient at this level since the nutrients were easier to get and degrade.

As a result of the growth of *P. ostreatus* on the manure for two months together with the storage conditions of the pile, without drainage and covered with a black canvas, there were some significant differences in methane production depending on the depth of the layer. The lower layer was found to yield significantly ($p < 0.01$) higher cumulated methane (249 ± 12 mL CH$_4$ gVS^{-1}), producing 111% more methane than the control (118 ± 6). Meanwhile, the upper layer produced 114 ± 4 mL CH$_4$ gVS^{-1} respectively with no statistical significance with the control stored at 4 °C (3A). The degradability of the lower layer substrate was 83% comparing the experimental results to the theoretical ones, while both the upper layer and the control presented a degradability of 39% (Table 3), like control and *P. ostreatus* pre-treatment in the

short-term assay. The presence of fungi in the process made the nutrient transportation a key factor to allow fungal growth. The fungus depends on moisture content, as well as the rest of microorganisms involved, so enough moisture ensures the growth of fungi and their ligninolytic activity. Moisture also had an important effect on the lignocellulosic fraction of the manure mixed with the cattle bedding by the absorption of water, which reduces the inner cohesive forces, swelling the crystalline cellulose structure and making it more accessible to enzymes [39]. So, the lignocellulosic material of the manure in the lowest layer absorbed more water due to the leaching from the upper layers, which made it softer and more accessible to microorganism and enzymes [52]. Besides, *P. ostreatus*, selectively degraded lignin facilitating the subsequent transformation of carbohydrates into methane [53]. Another process could have occurred at the same time, an inverted phase

fermentation. The manure might have produced some biogas in the lower layer, mainly CO_2 as a result of the early stages of hydrolysis and acidogenesis by anaerobic bacteria. The gas bubbles could have attached to the manure making it float on the liquid that cumulated at the bottom splitting in two phases, a solid one with manure and a liquid one composed of all the leachates from the upper levels holding easily degradable nutrients, as [54] stated working with sludge. That process improved some operational parameters as well as reduced *E. coli* presence. Those processes could explain the increment in methane yield of manure of the deepest level as well as the higher values of some parameters at initial stage like SCOD or VFAs.

The daily production of methane in this assay can be seen in Fig. 3B, the curves showed that the lower layer was clearly yielding higher volumes of methane than the rest of the samples from the very beginning of the assay, yielding 73% more methane than control at day 1.

4. Conclusions

The pre-treatment with the crude enzymatic extract of extracellular enzymes of genus *Pleurotus* improved the biodegradability and methane production of livestock manure mixed with the animal bedding. The enzymatic pre-treatment is a cheap and environmentally friendly way to improve the methane production from lignocellulosic materials. More research should be done to have a deeper insight of this promising strategy.

P. ostreatus pre-treatment also improved the degradability and methane yield of cattle manure mixed with the animal bedding by anaerobic digestion in both lab and mesocosm scale, being the latter where the methane production resulted higher due to the combination of fungal treatment and all the processes that occurred due to pile conditions.

The conditions of storage of the manure, which allow the inverted fermentation phase, influenced the methane production. During the storage, two layers were created in the manure pile. The lower layer showed more nutrients and moisture than the upper one, favouring the fungal performance on lignin and thus, facilitating hydrolysis phase by anaerobic bacteria.

Genus *Pleurotus* and its extracellular enzymes together with storage management are key factors in biomethanization to improve methane production from an inexhaustible source like livestock manure, in a sustainable and environmentally friendly way.

Author contributions

Conceptualization, BM, NC, EE; methodology, BM, NC, ; software, BM, RAH; validation, CGD, LD, CE and RM.; formal analysis, BM, RAH; investigation, BM, RAH, CGD, NC, LD, CE, RM, EE; resources, RM, NC, ; data curation, BM, RAH; writing—original draft preparation, BM, RAH, EE; writing—review and editing, BM, LD, CGD; visualization, BM, EE, NC, RM; supervision, EE, NC, RM; project administration, EE, BM, RAH; funding acquisition, EE, BM. All authors have read and agreed to the published version of the manuscript."

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Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data, and in the writing of the manuscript.

Data availability

Data will be made available on request.

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